ANNUAL PROGRESS REPORT

Report prepared by: Helen Simpson Vishniac Date: 1 January 54
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ANNUAL RATE: $5,822.00

CONTRACTOR: Yale University

PRINCIPAL INVESTIGATOR: Helen Simpson Vishniac
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TITLE OF PROJECT: Research in Marine Fungi

Objectives:
(1) Development of a metal-buffered artificial sea water and a more defined medium to permit harvesting large quantities of Labyrinthula cells.
(2) Development of a completely synthetic medium for Thraustochytrium and determination of its response to cobalamin.
(3) Determination of the specificity of the steroid requirement of Labyrinthula vitellina and
(4) isolation of the steroids in different strains of Labyrinthula and investigation of their role in the metabolism of the cell.

SUMMARY OF RESULTS:

(a) Since start of project: See below.

(b) During current report period: 1) Thraustochytrium does not require cobalamin.

2) Three strains of Labyrinthula can use amino acids as substrates. The small atlantic strain requires L-Leucine as growth factor in the presence of proline or alanine as substrate. All three strains require thiamin.

3) The method of assaying steroids for L. vitellina var. pacifica activity has been improved. A number of additional steroid preparations have been tested. $\Delta^8(14)$-stenols and products of mild oxidation of cholesterol represent new types of compounds found to be active.

4) Labyrinthula may be grown in liquid media on a rotary shaker or more conveniently, but less efficiently, in air-stirred carboys. Approximately 12.2 g. of wet small atlantic L. cells yielded 3.0 mg. of a sterol slowly active in the Moore-Bauman 3 B-OH sterol test.

PLANS FOR FUTURE:

Immediate: Further development of defined media for Thraustochytrium and three strains of Labyrinthula with particular attention to inorganic requirements, i.e., development of a metal-buffered artificial sea-water. Completion of study of amino acid requirements of Labyrinthula.

Development of methods for the detection of microquantities of pacifica-active types of steroids. Isolation and identification of pacifica-active steroids synthesized by other strains of Labyrinthula.

Feeding of labeled steroids to pacifica strain and recovery of products with the aim of identifying the steroid metabolite active in the cell.

Long Range: The topics intended for immediate investigation reflect two main interests: 1) in the nutritional determinants of marine microbial ecology. 2) in cellular biochemistry, as investigated by means of nutritional experiments. Marine microorganisms are so little touched that it is safe to predict generally interesting biochemical results from almost any investigation of their nutrition: in the present case they offer unique material for the identification of an essential steroid metabolite, doubtless also essential for other sorts of cells. Future investigations will probably continue to reflect these interests, utilizing other marine microorganisms.

REPORTS AND PUBLICATIONS: none

Detailed progress of investigation:

Thraustochytrium: Numerous experiments conducted before the effective date of this contract failed to confirm the cabalamin requirement of this fungus. Since preliminary experiments had indicated that both thiamine and cobalamin were required, it is difficult to explain the apparent cobalamin requirement on the basis of contamination of that solution with thiamine. The precautions found necessary in other laboratories for the demonstration of cobalamin requirements were taken in the later series of experiments. A possible explanation of the discrepancy in the two series of experiments may lie in the differences in mineral contents of the basal medium, which was constantly undergoing improvements. Bardos and Gordon (1953, JACS 75(8):2019-2020) found that ionic inhibition of Lactobacillus leichmannii 313 by a variety of salts could be reversed by the addition of cobalamin above the normal growth requirement. Further work on a defined Thraustochytrium has awaited completion of a rotating test tube apparatus (now ready); work during the period of this report was accordingly confined to

Labyrinthula: (1) Development of defined media: The first requisite for the development of a defined medium is the discovery of a substrate available in purified form at minimum cost. Experiments conducted prior to the effective date of this contract failed to uncover such a substrate for Labyrinthula strains. Amino acids are not - because of difficulties of purification from traces of other amino acids and inorganic material - substrates of choice for such work. Since Labyrinthula persisted in disregarding my preferences, amino acids were tested as substrates for growth of the three strains. The medium used consisted of a mineral base (essentially as published: Vishniac and Watson, 1953. J. Gen. Microb. 8 (2): 248-255), agar (0.11%), and thiamine. HCl (10-20 %). Us of a large inoculum taken directly from stock culture on 0.2% gelatin hydrolysate permitted growth on the following amino acids (0.05%), given in order of decreasing effectiveness:

Small atlantic strain: (gelatin hydrolysate), L-leucine, DL-Alanine, L-Proline, L-Glutamic acid.
Pacific strain (with cholesterol 1.0 mg. %): L-Glutamic acid, L-Proline, (gelatin hydrolysate), DL-Alanine, L-Arginine.
Atlantic strain A-1: (gelatin hydrolysate), L-Glutamic acid, L-Aspartic acid, DL-Alanine, L-Proline.

The small atlantic Labyrinthula was then serially subcultured with each of the amino acids used as substrate in the above experiment. In each series growth ceased or was materially decreased (L-Leucine) in the second subculture. The L-Leucine series was not carried further. Growth in the second subculture on L-Proline was restored to a high level only by the addition of L-Leucine (10 mg. %) of 16 amino acids tested. This level was not substantially improved by the further addition of any single amino acid and only slightly by gelatin hydrolysate in the third subculture. A dose response curve for L-Leucine (in the presence of L-Proline as substrate) was obtained, confirming the leucine growth factor requirement. In this experiment 10 mg. % of L-Leucine was slightly inhibitory; 20.0 mg. % of L-Leucine begin to inhibit growth even in the presence of 0.01% gelatin hydrolysate. L-Leucine is obviously not a satisfactory substrate. Second subcultures on DL-Alanine responded as on L-Proline and are being taken through third subculture to fourth subculture in an experiemtn designed to give a dose response curve for the L-Leucine requirement. The behaviour of Labyrinthula on NaHGlutamate is somewhat more complicated as this amino acid is slightly inhibitory in substrate concentration. It seems worthwhile to carry this substrate to the same stage as the others(1) to confirm the requirement for L-Leucine as growth factor, (2) to show that neither proline, nor alanine, nor glutamic acid is required as a growth factor, and (3) to demonstrate, if it is economically possible, the factors involved in increased growth with the addition of gelatin hydrolysate. The maundering quality of the usual explanation (that the use of a variety of performed building blocks saves the energy of synthesis, and anyway it's more "natural") is obvious from the fact that 7 of the 16 amino acids used in these experiements gave evidence of toxicity in growth factor concentrations. Balance, rather than mere variety, appears to be involved - e.g. the toxicity of valine may be overcome by the addition of a fixed ratio of isoleucine as in Bonner's Neurospora experiements.

Subcultures of the pacific and atlantic A-1 strains will be carried sufficiently far to define their amino acid growth factor requirements.

All three strains require thiamin work performed since publication (Vishniac and Watson, 1953. op. cit.) but before the effective date of this contract).

The mineral base used is adequate for large scale growth of Labyrinthula with gelatin hydrolysate as substrate. Since there are interesting differences between the strains, further work on the salt requirements, utilizing the rotating apparatus referred to under "Thraustochytrium", is planned.

(2) (Task order Section A (3)) the specificity of the steroid requirement of Labyrinthula vitellina var. pacifica. Since publication on this topic (Vishniac and Watson, 1953. op cit.), several minor changes in the method of steroid assay have been made, improving the sensitivity and reliability of the assay:

(1) Instead of a mixture of vitamine, 20% of thiamine. HCl are used,
(2) gelatin hydrolysate has been increased to 0.2%,
(3) steroids are added as freshly prepared ethanolic solutions, serially diluted so that 1 drop of solution/ 10 ml. medium is used. Addition is made after the basal medium has been autoclaved. Although steroid solutions are not sterilized, no contaminations have occurred.
(4) inoculation is made directly from stock cultures, using 1 drop/ 10 ml. of medium.

Generous gifts of steroid by various investigators, mainly Dr. W. Bergmann of this university and Dr. L.F. Fieser, have made possible marked advances in characterization of active types of compounds. The main difficulties which have been encountered are 1) many compounds are avialable only as esters, which must be saponified, and purified before use, 2) many compounds have degenerated since preparation and must be several times recrystallized before use - since only a few milligrams are available of some of these, this has constituted a major difficulty. Useful chromatographic methos for separation of sterols are unfortunately not available.

The following compounds have been tested (in order of decreasing activity):
Oxidation products of cholesterol: cholestenone (recrystallized Matheson, Coleman and Bell; Concrod Laboratories); Cholestane-3B, 5a, 6B-triol, Δ^4-cholestene-6B-ol-3-one, cholestane-3, 6-dione (Fieser);
7-keto-cholerterol (Rosenkrantz);
Oxidation products of cholesterol: ^{4}-cholestene-3, 6-dione; Δ5-cholestene-3-one (Fieser);
Δ8(14)-stenols: α-ergostenol (rosenkrantz); χ-stellastenol (Bergmann); Δ8(14)-cholestenol (Idler);
cholesterol (Pfanstiehl, as received);
7-dehydrocholesterol (Langdon)*
Δ5-sterols: haliclonasterol*, palysterol*, campesterol*, clionasterol* (Bergmann);
Δ5,22-sterols: poriferasterol*, (Bergmann); stigmasterol (recrystallized from Glidden Co. by van Wagtendonk, Paramedium active)
Δ-sterols: lathosterol (Fieser) showed slight activity, γ-ergostenol (Berstein) none (slightly inhibitory during part of range tested).
Δ7,22-sterols (spinasterol), Δ14,(15)-sterols (B-stellastenol*, B-ergostenol*), C-24 oxidized sterols (24-keto-cholesterol, cerebrosterol, cerebrostenolone), broken ring steroids (Windau's keto acid*, Diel's acid), saturated steroids with one or no hydroxyl functions (ergostane*, cholestane*, ergostanol*), non-steroid precursors(squalene*) and contaminants (Fieser's "lupid diol" of cholesterol were inactive. Some of these compounds were highly toxic.

* Indicates compounds tested before the period covered by this report, using assay methods not now standard, but since publication.

The activity of the products of mild oxidation of cholesterol, and of the Δ8(14)-stenols represents a major new development.

(3) (Task order section A (4) isolation of the steroids in different strains. The existence of a steroid growth factor requirement in the pacific strain of Labyrinthula implies that related forms without such a requirement synthesize one or more steroid metabolites. The following work has been done toward isolating and identifying steroids produced by the small atlantic A-1 strains: Prior to the period covered by this report 10 liters of small atlantic

Labyrinthula were grown on a rotary shaker in small lots, the cells harvested and stored in a dry ice chest. Approximately 12.2 grams of wet cells were obtained. These have now been saponified under nitrogen in methanolic KOH, diluted with two volumes of water and extracted continuously with ether. The ether extract was reduced under nitrogen to 20-30 ml, then dried in a vacuum dessicator with silica gel. The residue was suspected to contain a sterol or a compound of the type of cholestonone (at the time other types of compounds were not known to be active). The Moore-Baumann modification of the Lieberman-Burchard (Sperry-Schonhoimer) test for cholestorol indicated the presence in the total residue of 3.0 mg. (ca. 0.0246 % of wet weight) slow-acting 3-B hydroxy sterol. The residue proved too toxic for assay with the pacific strain. The remainder of the small atlantic sterol is stored as the digitonide, awaiting the accumulation of sufficient material for purification, identification, and biological assay.

It is proposed to test an aliquot of this residue for cholestenone, using a modification of the Gornall-MacDonald procedure which should render this test specific for the identification of Δ^4 -sterene-3-ones. The major difficulty encountered in the use of this test has been a floc appearing during the reaction which makes it impossible to get accurate colorimeter readings.

The detection of microquantities of other types of steroids now known to be active for the pacific Labyrinthula is being studied.

Approximately 26 liters of atlantic A-1 Labyrinthula have been grown in 5 and 10 liter lots in a carboy stirred by air bubbles. This method does not give as large a yield per unit volume as growth on a rotary shaker but it is more convenient. The harvested cells are now in storage in dry ice.

I noticed with interest (and pleasure, since this simplifies matters) that the extract of small atlantic cells contained no carotenoids. While many organisms share this feature with Labyrinthula, few of them are pink as Labyrinthula is in old cultures or in bulk. The small atlantic strain, which does not grow on tyrosine or phenylalanine, colors the medium pink in the presence of these amino acids - suggesting that phenylalanine can be oxidized to tyrosine and that the pink color may be due to dopachrome.